IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	:	Doddabele L. Madhavi, *et al.*
Serial No.	:	10/735,335
Filed:	:	December 12, 2003
For:	:	Bioavailable Carotenoid-Cyclodextrin Formulations For Soft-Gels And Other Encapsulation Systems
TC/AU	:	1623
Examiner	:	Leigh C. Maier
Attorney Docket No.	:	BIO 2-016

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DECLARATION UNDER C.F.R. § 1.132

Doddabele Madhavi, does declare and state that:

1. She received a Bachelor of Science degree in Biology and Chemistry in 1977 from University of Mysore, Mysore, India; a Master of Science degree in Botony in 1979 from Mysore, Mysore, India, Majors: Plant Biochemistry, Physiology, and Genetics; and a Doctorate degree (Ph.D.) in Biochemistry in 1987 from Central Food Technological Research Institute, Mysore, India, Thesis: Studies on the effects of processing on amino acid availability and functional properties of vegetable proteins.
2. She was a Research Fellow at the Department of Protein Technology, Central Food Technological Research Institute, Research Focus: Physico-chemical properties of vegetable proteins. Mysore, India, '80 to '87; a Research Scientist at the Department of Fruit and Vegetable Technology, Central Food Technological Research Institute. Research focus: Phytochemicals in cell cultures of food crops. Mysore, India, '88 to '90; a Research Associate at the Department of Nutrition and Food Sciences, Utah State University. Research focus: Color stability in bovine skeletal muscles. Logan, UT, '91 to '93; a Research Associate at the Departments of Food Science and Horticulture, University of Illinois, Research focus: the presence and use of flavonoids in crop plants and cell cultures. Urbana, IL, '93 to '96; a Visiting Assistant Professor at the Department of Natural Resources and Environmental Sciences, University of Illinois, where she researched the screening, extraction and formulation of bioactive compounds from novel

sources. Urbana, IL, '96 to 98; Chief Scientist for PhytoLife Sciences, Inc. where she provided a mid-course evaluation of a novel technology to produce natural product for a company in a turn-around situation. With management, developed enhancements and alternatives to the technology to meet pressing needs to generate revenues. Succeeded in redirecting the Company's conceptual basis while still maintaining its core value. Responsible for all laboratory functions Columbus, OH, '98 to '99; and currently is Managing Partner for BioActives LLC, the assignee of the above-identified application where she provides the scientific expertise for new product development, process development and scientific/experimental strategies therein. In collaboration with management chart the company's strategic direction. Responsible for all laboratory functions including but not limited to the concept, experimental design and execution, ordering equipment and supplies, and personnel and environmental safety. Worcester, MA. Oct. '99 to present.

3. A more complete resume for her is attached hereto.

4. She is a co-inventor of and co-applicant for the invention described and claimed in the above-identified application.

5. She has read an Office action dated January 7, 2008, and the art cited therein and as applied in a rejection of the claims.

6. Mele teaches the preparation of a β-carotene-γ-CD complex. The solid mixture contained 9.4% w/w β-carotene. Based on the initial weights used, the complex contains 1:4 molar ratio of β-carotene to cyclodextrin. After complexation and drying, Mele did not determine the amount of β-carotene in the complex. However, they determined the amount of uncomplexed β-carotene to be 0.2% w/w. This indicates a high degree of complexation.

7. **Complexation of Lutein with γ-CD by the Protocol of Mele.**

Commercially available crystalline *all trans*-lutein (70% purity, manufactured under U.S. Patent No. 6,380,442) was used for complexation. 0.77g of free lutein (equivalent to 0.54g pure lutein) was mixed with 1.95g γ-CD (1:2 molar ratio lutein:γ-CD) in a mortar and kneaded until a uniform powder was obtained. Kneading was continued after adding 16ml of water to form a slurry. The mixture was allowed to stand overnight under nitrogen and then it was resuspended in 1.6L of warm distilled water (40˚C). The suspension was stirred for 20 min at 40˚C and filtered under vacuum using a standard filter paper.

Results and Observations:

It was observed that most of the material was retained on the filter paper and a pale yellow filtrate was obtained.

The filtrate on freeze-drying resulted in 1.22g of a pale yellow powder containing 1.46% lutein. The molar ratio of lutein to γ-CD was 1:29.6. The expected product yield was at least 2.4g, assuming a 10% loss during processing. The expected lutein percentage was at least 19.9%, based on the initial weights used for the experiment.

The protocol of Mele does not disclose the yield of the final product. Based on the description, one is lead to assume that they obtained a fully water-soluble complex that can pass through the filter paper. (see Abstract, for example).

Applicants have described a commercial process for making the complex with 95% recovery of the product containing 20% lutein at a molar ratio of lutein to γ-CD of 1:2.

The results from using the Mele process clearly show that application of the protocol to complex lutein with γ-CD results in very poor complexation and cannot be used on a commercial scale.

Characterization of the Complex Using Differential Scanning Calorimetry (DSC)

The product was further characterized using Differential Scanning Calorimetry in comparison to the complex produced using the Applicants method.

Conventional analytical methods such as spectrophotometry or HPLC require the complex to be dissolved in solvents, which results in a dissociation of the guest molecule from the cyclodextrin. Hence, solid-state analytical techniques are used to characterize the complexes in comparison to the free cyclodextrin and the guest molecule. DSC is one such technique widely used to characterize the cyclodextrin inclusion complexes (US Patent 6,984,632; US Patent Application 20040176265, Orgovanyi, J., Poppl, L., Otta, K.H., and Lovas, G.A., Thermoanalytical method for studying the guest content in cyclodextrin inclusion complexes, *J. Thermal Analysis and Calorimetry*, 81: 261-266, 2005). DSC allows for the determination of the complexed and uncomplexed guest molecule. The ratio of the area of the endotherm of the guest molecule (GM) before and after complexation is used to calculate % uncomplexed guest molecule [δ-H (j/g)$_{\text{free GM}}$/δ-H (j/g)$_{\text{pure GM}}$ X 100 = % uncomplexed GM].

DSC studies were conducted by an independent testing laboratory. Figures 1 to 4 represent the DSC thermograms of γ-CD, free lutein, lutein-γ-CD complex made using the inventive method, and lutein-γ-CD complex made using Mele protocol, respectively. All the samples were passed through 60 mesh sieve before the study. The reported

melting point of lutein is 183°-190°C and the thermogram of free lutein has an endotherm at 182.96°C.

The complex made using the inventive process shows a reduced area for the lutein endotherm, as compared to the free lutein thermogram. The ratio of the two areas 0.5074/4.152 x100 = 12.2% is the uncomplexed lutein in the complex. This indicates nearly 88% of the lutein is in a complexed form.

The Mele complex shows complete disappearance of the lutein endotherm, indicating that lutein was fully complexed with the cyclodextrin. The complex has 1.46% (w/w) lutein as determined by spectrophotometry. So 1.22g of the complex contains 17.8mg of lutein. This leaves ~ 96% of the lutein uncomplexed by using this process.

Figure 1. DSC Thermogram of Free Lutein

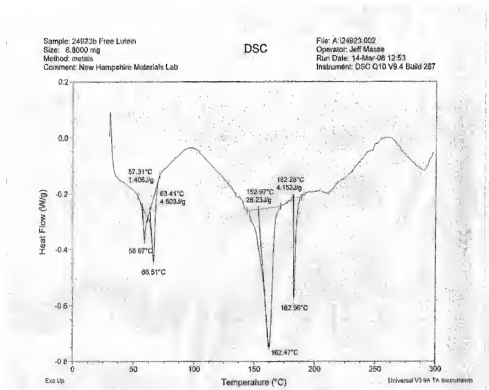


Figure 2. DSC Thermogram of γ-CD

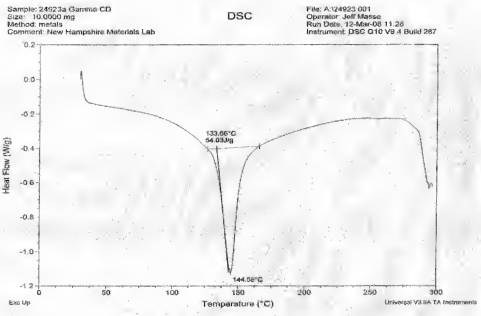


Figure 3. DSC Thermogram of Freeze-Dried Lutein-γ-CD Complex

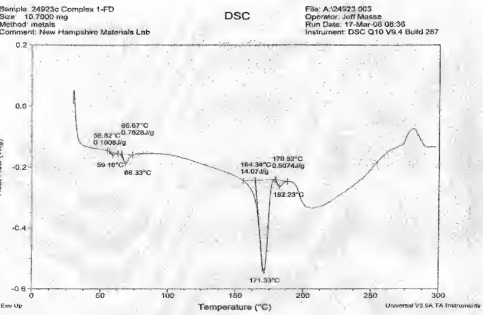
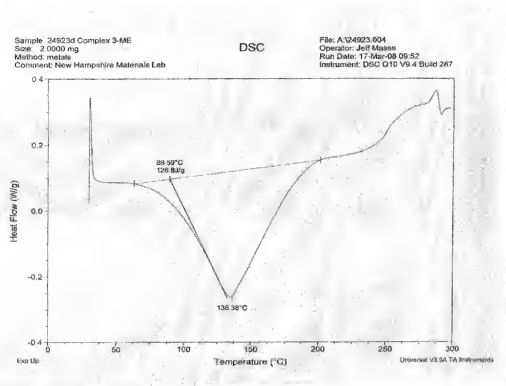


Figure 4. DSC Thermogram of Spray-Dried Lutein-γ-CD Complex



8. **Characterization of Freeze-Dried and Spray-Dried Lutein-γ-CD Complex**

It is known in the art that formation of cyclodextrin inclusion complex is sensitive to the process parameters, such as, for example, complexation method used (kneading, dispersion, melting in solution to name a few), solvents used, energy input in terms of heat, high shear homogenization, and drying method. Such variables are not described, nor are their effects obvious, in the teachings of Mele, Pfitzner, or Szente.

As an example, the effect of drying on the properties of the lutein-γ-CD complex was determined. The freeze-dried and spray-dried lutein-γ-CD complexes were characterized by analysis of particle size in aqueous dispersion and DSC. The analyses were done by independent testing labs.

1. Particle Size Analysis in Aqueous Dispersion

The analysis was done using optical microscopy. The behavior of the two samples in aqueous dispersion was determined. The study also was expected to shed some light on the differences observed in the uptake in the human study. The lutein-γ-CD complex was prepared in aqueous slurry, as described in the Application, and split into two equal

sized lots for spray-drying and freeze-drying. After drying, the samples were passed through 60mesh for the study.

Samples Tested:

Sample#1. Freeze-dried lutein/γ-CD powder

Sample#2. Spray-dried lutein/γ-CD powder

HIGHLIGHTS OF THE STUDY

The results of the study indicate the freeze-dried complex formed a suspension in water fairly easily, as compared to the spray-dried complex. The spray-dried complex also showed a tendency to form aggregates in water. This may in part explain the differences in the uptake observed between the two products.

Report from the Testing Lab

Summary

Lower apparent particle density in suspension combined with larger average particle size and rough "lumpy" particle morphology suggests that Sample #2 agglomerates more strongly than Sample #1.

OPTICAL MICROSCOPY

Two orange powder materials were supplied for particle size analysis; Sample #1 Complex 1-FD and Sample #2 Complex 2-SD. A few milligrams of each were mixed into a few milliliters of distilled water in order to suspend the particles and allow for agglomeration. Sample #1 went into suspension fairly easily. Sample #2 required additional stirring and agitation, however; the powder was not as easily wet.

Once the solutions were prepared, a few drops of each were placed on a clean glass microscope slide between two clean glass cover slips. A second clean glass microscope slide was placed on top of the cover slips causing the drops of solution to join and spread in the space between the cover slips. The solution filled the space from top to bottom, and the cover slips maintained a solution thickness sufficient to allow motion and agglomeration of the suspended particles. The prepared specimens are shown in Figure 1. By trapping the prepared solutions in this manner the particles could be viewed from the top to the bottom of the reservoir.

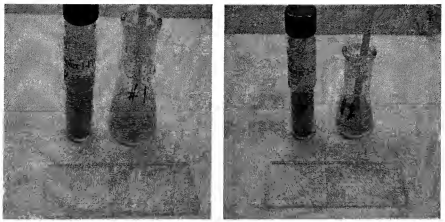


Figure 1. A few milligrams of each specimen were suspended in a few milliliters of water. A few drops of the resulting solution were trapped between two cover slips and sandwiched between two glass slides. The resulting solution could be examined from top to bottom.

The glass slides were individually mounted on a metallographic microscope such that viewing took place through the bottom slide. The specimens were backlit. In the collected images of Sample #1the orange material often appeared to be thin and flake-like, with sharp and angular edges. These flakes were primarily found at the bottom of the reservoir. Fewer individual particles were seen at the top of the reservoir, and those found tended to have rougher more complex edges. These particles seemed to have suffered agglomeration more than those at the bottom of the reservoir.

The collected images of Sample #2 were somewhat similar to those of Sample #1, mostly in that a higher density of particles could be found at the bottom of the reservoir than at the top. Qualitatively comparing Sample #1 with Sample #2 there appeared to be a lower density of suspended particles in Sample #2. Furthermore, there appeared to be greater particle agglomeration in Sample #2. Sample images are shown in Figure 2. A scale image is also provided. Figure 3 shows a sample image for each suspension at higher magnification. These were taken from the bottom of the reservoir in each case.

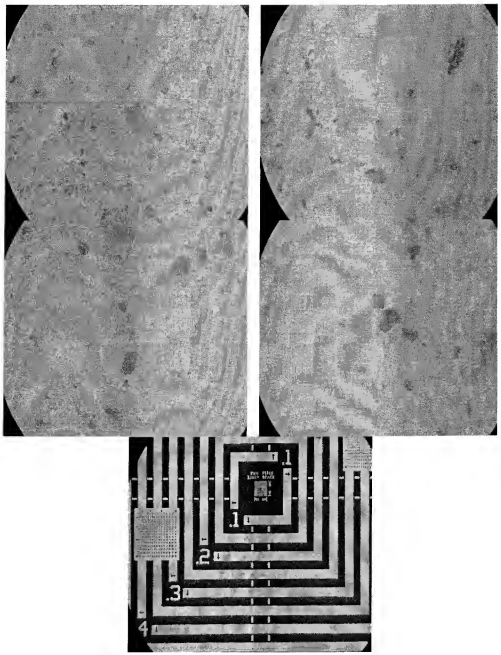


Figure 2. Top: Sample #1 bottom (left) and top (right) of the reservoir. Middle: Sample #2 bottom (left) and top (right) of the reservoir. Bottom: Scale image – bars and spaces each represent 25 μm.

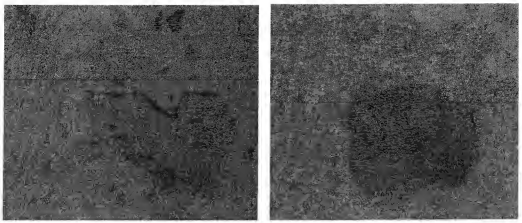


Figure 3. Higher magnification images of relatively large particles and the surrounding area from Sample #1 (left) and Sample #2 (right) are shown. Both were taken at the bottom of the reservoir. The typical particle in the Sample #1 suspension was thin and angular with sharp edges. The particles in the Sample #2 suspension were often more rounded and "boulder-like" in appearance. These images suggest greater agglomeration for Sample #2.

2. Differential Scanning Calorimetry (DSC)

Figures 1 and 2 present the DSC thermograms of freeze-dried and spray-dried lutein-γ-CD complex respectively. Please see page 3 for the DSC theromogram of free lutein. The spray-dried complex surprisingly does not show the endotherm around 182°C, the melting point of free lutein. The spray-dried complex also shows a shift in the major endotherm to a lower temperature. The freeze-dried complex has a free lutein endotherm, with highly reduced area. As described before, nearly 88% of lutein is in a complexed form in the freeze-dried complex. The results indicate that on spray-drying, there is a change in the degree of complexation and possibly a change in the physical properties of the complex.

Figure 1. DSC Thermogram of Freeze-Dried Lutein-γ-CD Complex

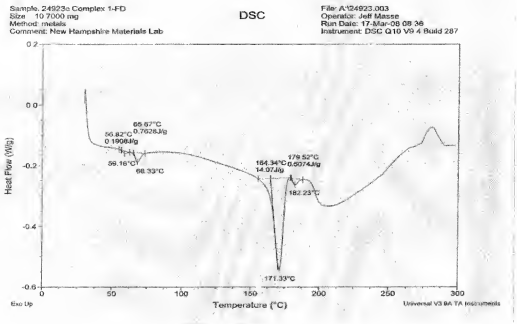
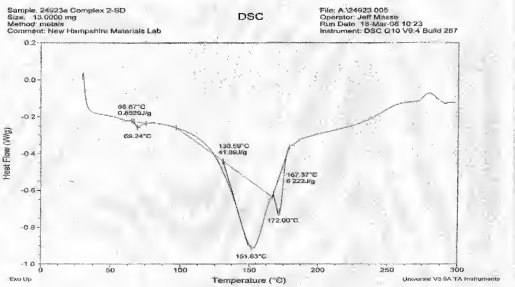


Figure 2. DSC Thermogram of Spray-Dried Lutein-γ-CD Complex



8. **Response to Examiner's Comments on Bioavailability of the Lutein/γ-CD Complex Without Excipients**

A small crossover human study was conducted to demonstrate the following:

1. Uptake of the lutein/γ-CD powder without any excipients
2. Compare the uptake of spray-dried and freeze-dried lutein/γ-CD complex without any added excipients.

Protocol

The protocol described in Example 6 was followed for the study. A single dose of 100 mg lutein was used for the study with two volunteers. The lutein-γ-CD complex was prepared in aqueous slurry as described in the Application and split into two equal sized lots for spray-drying and freeze-drying. After drying, samples were passed through 60mesh for the study.

Samples Used

1. Freeze-dried lutein/γ-CD powder in hard gelatin capsules
2. Spray-dried lutein/γ-CD powder in hard gelatin capsules

Subjects

The subjects were non-smokers, without any chronic diseases or gastrointestinal disturbances. The subjects were not taking any over-the-counter carotenoid supplements and were on their usual diets with modest amounts of carotenoids during the study.

Method

On the day of the study, 10 ml of blood was drawn to permit establishment of baseline values. After consumption of single doses of lutein from one of the formulations, blood samples were drawn at 24 hours. Serum was separated within 1 hr of blood draw and frozen immediately. After a two-week washout period, the study was repeated with the second formulation. The blood samples were drawn and processed as before.

Lutein and other carotenoids were extracted from the serum using Khachik's method (Khachik, F., Beecher, G.R., Goli, M.B., Lusby, W.R., and Daitch, C.E., "Separation and quantification of carotenoids in human plasma", *Methods in Enzymol.*, 213: 205-219, 1992). The lutein concentration was determined using reverse phase HPLC and diode array detection. The extraction method used sufficient serum for the detection of carotenoids by the diode array detector. The area under the curve was used for calculations.

Observations

The results recorded are displayed below.

Subjects	Serum Lutein Percent Increase (Treated/Baseline)	
	Freeze-dried Lutein complex	Spray-dried Lutein Complex
1	85	17
2	59	18

The results clearly show that the freeze-dried complex is more bioavailable than the spray dried complex *in vivo*. Furthermore, the lutein from the complex is bioavailable without any added excipients *in vivo*.

Excipients are needed to improve the shelf-life of the complex to meet industry specifications. Applicants statement, "the carotenoids are not completely protected from degradation by the complexation, [so] further formulations are necessary for incorporation into soft gelatin capsules", pertains to the shelf-life of the complex. In the powder form, the complex in hard gelatin capsules has a shelf-life of approximately six months, while the industry standard requires a minimum of two years shelf-life.

9. **Response to Examiner's Comments on the Degradation of Lutein Before Absorption**

Lutein is known to be stable in the presence of food matrix components such as fats, proteins, starches, phytochemicals and alkaline pH. However, *in vivo*, lutein also is exposed to the acidic pH of the gastric fluid. The objective of this experiment was to demonstrate the stability of lutein complexed with γ-CD on exposure to simulated gastric fluid.

Method:

Varian USP Apparatus #1 (basket), 75rpm, 37°C.

Dosage Tested: Complex equivalent to 100mg lutein.

Samples Preparation: The lutein-γ-CD complex was prepared in aqueous slurry as described in the Application and split into two equal sized lots for spray-drying and freeze-drying. After drying, the samples were passed through 60mesh for the study.

Samples Tested

1. Freeze-dried lutein/γ-CD powder in hard gelatin capsules
2. Spray-dried lutein/γ-CD powder in hard gelatin capsules

Dissolution Medium : Simulated gastric fluid (USP), 0.1N HCl, 500ml, pH 1.20

Duration: 1hr to simulate gastric residence time

At the end of the run, the lutein-CD complex was collected by vacuum filtration. The lutein content was determined by spectrophotometry and the percentage of lutein was calculated based on the dry weight of the samples.

Spectrophotometry: To 100mg of the lutein CD complex, was added 10ml of DMSO and the complex dissolved by warming to 37°C. To the solution 10ml of chloroform was added to completely dissolve lutein. The solution was diluted 1:100 with ethanol and the absorbance was determined at 446nm. An E1% of 2550 was used to calculate the % lutein (w/w)

Observations

The results recorded are displayed below

Sample	Starting Lutein %	% Lutein at the end of the run
Freeze-dried Lutein-CD complex	18.70	18.60
Spray-dried Lutein-CD Complex	18.60	18.45

The results demonstrate that both the complexes remain stable at pH 1.2 for the duration the ingested food or supplement is exposed to acidic pH in the stomach.

10. It is her expert opinion that the subject matter disclosed and claimed in the above-identified patent application is new, novel, and non-obvious over the cited art.

11. All statements made herein of her own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing therefrom.

FURTHER DECLARANT SAYETH NAUGHT.

4/2/08

Dt Madhavi
Doddabele Madhavi, Ph.D.

Resume
Doddabele Madhavi, Ph.D.

13 Schussler Road
Worcester, MA 01609
(508) 757-7726
lmadhavi@bioactives.com

BACKGROUND SUMMARY Over twenty years experience as a research scientist focused on exploring phytochemistry as it relates to human physiology and the development of natural-health enhancing products. Extensive experience in scaling up bench-top processes to produce commercial products.

Professional Experience and Selected Accomplishments

Professional Experience

Managing Partner, BioActives LLC

Provided the scientific expertise for new product development, process development and scientific/experimental strategies therein. In collaboration with management chart the company's strategic direction. Responsible for all laboratory functions including but not limited to the concept, experimental design and execution, ordering equipment and supplies, and personnel and environmental safety. Worcester, MA. Oct. '99 to present.

Chief Scientist, PhytoLife Sciences, Inc.

Provided a mid-course evaluation of a novel technology to produce natural product for a company in a turn-around situation. With management, developed enhancements and alternatives to the technology to meet pressing needs to generate revenues. Succeeded in redirecting the Company's conceptual basis while still maintaining its core value. Responsible for all laboratory functions Columbus, OH, '98 to '99

Visiting Assistant Professor

Department of Natural Resources and Environmental Sciences, University of Illinois, ---- Researched the screening, extraction and formulation of bioactive compounds from novel sources. Urbana, IL, '96 to 98

Research Associate

Departments of Food Science and Horticulture, University of Illinois, Research focus: the presence and use of flavonoids in crop plants and cell cultures. Urbana, IL , '93 to '96

Research Associate

Department of Nutrition and Food Sciences, Utah State University. Research focus: Color stability in bovine skeletal muscles. Logan, UT, '91 to '93

Research Scientist

Department of Fruit and Vegetable Technology, Central Food Technological Research Institute, Research focus: Phytochemicals in cell cultures of food crops. Mysore, India, '88 to '90

Research Fellow

Department of Protein Technology, Central Food Technological Research Institute, Research Focus: Physico-chemical properties of vegetable proteins. Mysore, India, '80 to '87

Patents Held and Filed

- U.S. Patent No. 6,380,442: "Process for the isolation of mixed carotenoids from plants", D.L. Madhavi, Daniel Kagan,
- U.S. Patent No. 7,030,102 A Highly Bioavailable Coenzyme Q-10 Cyclodextrin. D. L. Madhavi, Daniel Kagan.
- U.S. Patent No. 7,297,803, Process for the Preparation of Lutein Ester Concentrate. D. L. Madhavi, Daniel Kagan.
- U.S. Patent Application Ser. No. 10/309,999f for Coated Carotenoid Cyclodextrin Complexes, Helmut Schmidt, D. L. Madhavi, Daniel Kagan.
- U.S. Patent Application Ser. No. 11/344,375 (allowed) for the Preparation of Lutein Ester Concentrate. D. L. Madhavi, Daniel Kagan.
- U.S. Patent Application Ser. No. 12/070,195 for Water Dispersible Policosanol Cyclodextrin Complex and Method of its Production

Education

Doctorate – Biochemistry, 1987, Central Food Technological Research Institute, Mysore, India. Thesis: Studies on the effects of processing on amino acid availability and functional properties of vegetable proteins.
Master of Science – Botany, 1979, University of Mysore, Mysore, India. Majors: Plant Biochemistry, Physiology, and Genetics.
Bachelor of Science – Biology, Chemistry, 1977, University of Mysore, Mysore, India.

Selected Scientific Publications

Book

D.L. Madhavi, S.S. Deshpande, and D.K. Salunkhe, *Food Antioxidants: Technological, Toxicological, and Health Perspectives*, Marcel Dekker, New York, NY. (1996).

Book Chapters

D.L. Madhavi and D.K. Salunkhe, "Tomato", In *Handbook of Vegetable Science and Technology*. D.K. Salunkhe and S.S. Kadam (Eds.), Marcel Dekker, New York, NY, p 171-201. (1998).

P.M. Kotecha, B.B. Desai, and D.L. Madhavi, "Carrot", In *Handbook of Vegetable Science and Technology*. D.K. Salunkhe and S.S. Kadam (Eds.), Marcel Dekker, New York, NY, p 119-139. (1998).

S.P. Ghosh and D.L. Madhavi, "Cabbage", In *Handbook of Vegetable Science and Technology*. D.K. Salunkhe and S.S. Kadam (Eds.), Marcel Dekker, New York, NY, p 299-321. (1998).

D.L. Madhavi and S.P. Ghosh, "Cauliflower", In *Handbook of Vegetable Science and Technology*. D.K. Salunkhe and S.S. Kadam (Eds.), Marcel Dekker, New York, NY, p 323-336. (1998).

S.J. Jadhav, S.S. Nimbalkar, A.D. Kulkarni, and D.L. Madhavi, "Lipid Oxidation in Food and Biological Systems", In *Food Antioxidants: Technological, Toxicological, and Health Perspectives*, D.L. Madhavi, S.S. Deshpande, and D.K. Salunkhe (Eds.), Marcel Dekker, New York, NY, p 5-63. (1996).

D.L. Madhavi, R.S. Singhal, and P.R. Kulkarni, "Technological Aspects of Food Antioxidants", In *Food Antioxidants: Technological, Toxicological, and Health Perspectives*, D.L. Madhavi S.S. Deshpande, and D.K. Salunkhe (Eds.), Marcel Dekker, New York, NY, p 159-265. (1996).

D.L. Madhavi and D.K. Salunkhe, "Toxicological Aspects of Food Antioxidants", In *Food Antioxidants: Technological, Toxicological, and Health Perspectives*, D.L. Madhavi, S.S. Deshpande, and D.K. Salunkhe (Eds.), Marcel Dekker, New York, NY, p 267-359. (1996).

P.M. Kotecha and D.L. Madhavi, "Berries", In *Handbook of Fruit Science and Technology: Production, Composition, Storage, and Processing*. D.K. Salunkhe and S.S. Kadam (Eds.), Marcel Dekker, New York, NY, p 315-334. (1995).

D.L. Madhavi and D.K. Salunkhe, "Antioxidants", In *Food Additive Toxicology*, J.A. Maga and A.T. Tu (Eds.), Marcel Dekker, New York, NY, p 89-177. (1994).

Representative Scientific Publications

D.L. Madhavi, J.A. Bomser, M.A.L. Smith, and K. Singletary, "Isolation of bioactiveconstituents from *Vaccinium myrtillus* (bilberry) fruits and cell cultures". *Plant Sci.*, 131: 95-103. (1998).

J.A. Bomser, D.L. Madhavi, K. Singletary, and M.A.L. Smith, "In vitro anticancer activity of fruit extracts from *Vaccinium* species". *Planta Medica*, 62: 212-216. (1996).

D.L. Madhavi, S. Juthangkoon, K. Lewen, M.D. Berber-Jimenez, and M.A.L. Smith, "Characterization of anthocyanins from Ajuga pyramidalis 'Metallica Crispa' cell cultures". J. *Agric. Food Chem.* 44: 1170-1176. (1996).

D.L. Madhavi and C.E. Carpenter, "Aging and processing affect color, metmyoglobin reductase, and oxygen consumption of beef muscles". J. *Food Sci.*, 58: 939-942. (1993).

D.L. Madhavi, N. Chand, D. Rajalakshmi, and M.V. Patwardhan, "Effects of growth hormones and maturity of fruits on the callus culture of guava fruits using response surface methodology". *J. Sci. Food Agric.*, 58: 29-34. (1992).

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